

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
6 January 2005 (06.01.2005)

PCT

(10) International Publication Number  
**WO 2005/000318 A2**

(51) International Patent Classification<sup>7</sup>: **A61K 31/661**,  
A61P 35/00

(21) International Application Number:  
PCT/US2004/020104

(22) International Filing Date: 23 June 2004 (23.06.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/480,669 23 June 2003 (23.06.2003) US

(71) Applicant (for all designated States except US):  
NEOPHARM, INC. [US/US]; 150 Field Drive, Suite 195,  
Lake Forest, IL 60045 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **JAMIL, Haris**  
[US/US]; 1216 Trinity Place, Libertyville, IL 60048 (US).  
**AHMAD, Moglis, U.** [US/US]; 3050 North Forest Hills  
Court, Wadsworth, IL 60083 (US). **AHMAD, Imran**  
[US/US]; 4731 Pebble Beach Drive, Wadsworth, IL 60083  
(US).

(74) Agents: **HEFNER, M., Daniel et al.**; Leydig, Voit &  
Mayer, Ltd., Two Prudential Plaza, Suite 4900, 180 North  
Sielson Ave., Chicago, IL 60601-6780 (US).

(81) Designated States (unless otherwise indicated, for every  
kind of national protection available): AE, AG, AL, AM,  
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,  
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,  
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,  
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,  
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,  
PI, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,  
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,  
ZW.

(84) Designated States (unless otherwise indicated, for every  
kind of regional protection available): ARIPO (BW, GH,  
GM, KE, LS, MW, MZ, NA, SD, SI, SZ, TZ, UG, ZM,  
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,  
FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI,  
SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,  
GW, ML, MR, NI, SN, TD, TG).

**Published:**

— without international search report and to be republished  
upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: METHOD OF INDUCING APOPTOSIS AND INHIBITING CARDIOLIPIN SYNTHESIS

(57) Abstract: The present invention provides a method for inducing apoptosis within a cell by exposing the cell to an inhibitor of cardiolipin synthesis under conditions sufficient to induce apoptosis within the cell. The method can be used to investigate or treat disorders such as cancer, obesity, and cardiovascular disorders. The invention also provides a pharmaceutical composition including an inhibitor of cardiolipin synthesis and a liposomal carrier.

WO 2005/000318 A2

## METHOD OF INDUCING APOPTOSIS AND INHIBITING CARDIOLIPIN SYNTHESIS

### FIELD OF THE INVENTION

[0001] This invention pertains to a method of inducing apoptosis, principally via inhibiting the synthesis of cardiolipin, and therapeutic uses thereof.

### BACKGROUND OF THE INVENTION

[0002] Apoptosis, or programmed cell death, is an evolutionarily conserved mechanism of cell death that has a crucial role in various biological events, including development, the maintenance of homeostasis and the removal of obsolete cells [Reed, J.C. (1998) Bcl-2 family proteins. *Oncogene* 17, 3225 - 3236; Kroemr, G., Dallaporta, B. and Resche-Rigon, M. (1998), *Annu. Rev. Physiol.* 60, 619 - 642; Skulachev, V.P. (1998), *FEBS Lett.* 423, 275 -280]. Apoptotic signals are activated by various stimuli and converge towards a common death pathway, in which proteins in the Bcl-2 family act as regulators, and proteases in the caspase family act as signal transducers [Reed, J.C. (1998), *Oncogene* 17, 3225 - 3236; Kroemr, G., Dallaporta, B. and Resche-Rigon, M. (1998), *Annu. Rev. Physiol.* 60, 619 - 642; Skulachev, V.P. (1998), *FEBS Lett.* 423, 275 - 280]. Recent evidence has shown that mitochondria have a crucial role in apoptosis by releasing apoptotic factors such as cytochrome c and apoptosis-inducing factor from the intermembrane space into cytoplasm.

[0003] Apoptosis may occur by two general pathways, i.e. receptor-mediated and stress-induced (mitochondrial-initiated) apoptosis [Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999) *Annu. Rev. Cell Dev. Biol.* 15, 269 - 290]. In both pathways, cytochrome c release is one of the most important regulatory steps. In receptor-

mediated apoptosis, caspase 8 is activated early and cleaves BID [Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) *Cell* 94, 481 - 490]. After cleavage by caspase 8, the carboxy-terminal portion (tBid) moves from cytosol to mitochondria, where it induces release of cytochrome c. BID also appears to modulate lipid transfer between ER and mitochondria [Degli Esposti M et al (2001) *Mol Cell. Biol.* 21: 7268 - 7276].

[0004] In stress-induced apoptosis, however, caspase 8 is usually not activated, and the mechanism of cytochrome c release is uncertain. Current theories involve transient opening of the mitochondrial permeability transition pore causing slight swelling as well as formation of pores in the outer membrane by proapoptotic members of the Bcl-2 family, e.g. BAX and BAK [Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999) *Annu. Rev. Cell Dev. Biol.* 75, 269 - 290; Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V., and DiLissa, F. (1999) *Eur. J. Biochem.* 264, 687 - 701; Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) *Science* 292, 727 - 730]. These mechanisms mediate the passage of unbound cytochrome c through the mitochondrial outer membrane. Cytochrome c is bound to the outer surface of the inner membrane phospholipids by electrostatic forces (predominating at neutral pH). Dissociation from the inner membrane is a necessary first step before cytochrome c can pass through release of channels and ultimately reach the cytosol. The released cytochrome c activates caspase 9 in concert with the cytosolic factors ATP and Afaf-1, and, as a result, caspase 3 is activated [Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S. and Wang, X. (1997), *Cell* 91, 479 - 489]. Apoptosis-inducing factor has also been reported to induce apoptotic changes in the nucleus [Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J.,

Jacotot, E., Costantini, P., Loeffler, M. et al. (1999), *Nature (London)* 397, 441 - 446]. The anti-apoptotic proteins Bcl-2 and Bcl-xL, which are localized predominantly in mitochondrial outer membranes, inhibit the release of cytochrome c from mitochondria [Reed, J.C. (1998), *Oncogene* 17, 3225 - 3236]. Although cytochrome c normally shuttles electrons between complex III (cytochrome c reductase) and complex IV (cytochrome c oxidase) of the respiratory chain, cytochrome c released from mitochondria is an important proapoptotic signal [Kroemer, G., Dallaporta, B. and Resche-Rigon, M., *Annu Rev Physiol.* (1998), 60, 619 - 642; Skulachev, V.P. (1998), *FEES Lett.* 423, 275 - 280] in the mitochondrial death pathway.

[0005] The failure of cells to undergo programmed cell death is implicated in tumorigenesis in a variety of human malignancies. Cells that have accumulated high levels of DNA damage are eliminated from the organism via programmed cell death without negatively affecting the surrounding tissue. Disruption of programmed cell death in a cell greatly increases the chance of that cell becoming tumorigenic, since the damage can cause mutations that lead to malignant transformation. In addition, programmed cell death appears to be a first line of defense against the proliferation of cells that might form a tumor: cells in which growth control is dysregulated in a way that could result in uncontrolled proliferation are generally able to recognize that aberrant state and commit suicide by programmed cell death. If programmed cell death is blocked in such cells, cancer could arise. The failure to undergo programmed cell death per se can even lead to excessive number of cells and cancer: e.g., as the result of inappropriate activation of the Bcl-2 gene, a suppressor of programmed cell death, most follicular B cell lymphomas result in the accumulation of excessive number of cells that would normally undergo programmed cell death. Many tumor cell types also appear to require Bcl-2 expression to

avoid apoptosis and remain proliferative. Thus, the inability to regulate programmed cell death may be a key causative even in many, and perhaps all, cancers. Regulation of apoptosis also may be important for other disorders, such as obesity and cardiovascular disorders characterized by fatty plaque buildup in the walls of vessels. Thus, there is a need for methods for regulating apoptosis.

#### BRIEF SUMMARY OF THE INVENTION

[0006] The present invention provides a method for inducing apoptosis within a cell by exposing the cell to an inhibitor of cardiolipin synthesis under conditions sufficient to induce apoptosis within the cell. The method can be used to investigate or treat disorders such as cancer, obesity, and cardiovascular disorders. The invention also provides a pharmaceutical composition including an inhibitor of cardiolipin synthesis and a liposomal carrier. These and other advantages of the invention, as well as additional inventive features, will be apparent upon reading the following detailed description of the invention.

#### DESCRIPTION OF THE FIGURES

[0007] Figure 1 depicts the structure of cardiolipin

[0008] Figure 2 is a flowchart depicting the cardiolipin synthetic pathway.

#### DETAILED DESCRIPTION OF THE INVENTION

[0009] The invention provides a method of inducing apoptosis in a cell. In accordance with the inventive method, the cell is exposed to an inhibitor of cardiolipin synthesis under

conditions sufficient to induce apoptosis within the cell. Cardiolipin (Figure 1) is a unique dimeric phospholipid that contains four acyl groups and two negative charges. The name "cardiolipin" is derived from the fact that the compound was first found in animal hearts, where it is especially abundant. However, cardiolipin is found almost exclusively in mitochondria and bacteria, i.e. those whose function is to generate an electrochemical potential for substrate transport and ATP synthesis, and can account for as much as 20% of mitochondrial lipids [Pangborn MC, J Biol Chem 1942, 143, 247]. Cardiolipin accounts for about 10% of the phospholipids of bovine heart muscle. In animal tissues, cardiolipin contains almost exclusively 18 carbon fatty acids, and 80% of this is typically linoleic acid

[0010] Cardiolipin is a specific lipid component of mitochondria and its biological function in this organelle is clearly crucial. Cardiolipin is located mainly on the inner membrane of mitochondria, where it interacts with a large number of mitochondrial proteins, such as NADH: ubiquinone oxidoreductase, cytochrome. c oxidase and cytochrome c. This interaction effects functional activation of certain enzymes, especially those involved in oxidative phosphorylation. Indeed, many of the mitochondrial protein complexes contain cardiolipin molecules integrated into their quaternary structure, where they are essential components of the interface between the complex and its environment or between subunits within the complex. Removal of cardiolipin leads to the break-up of the complex and loss of functionality.

[0011] Cardiolipin is the most unsaturated lipid in the human body due to a process of constant re-modeling integrated between mitochondria and ER [Schlame M and Rustow B (1990) Biochem. J. 272: 589 - 595; Kajiyama, K., Pauly, D. F., Hughes, H., Yoon, S. B., Entman, M. L., and McMillin-Wood, J. B. (1987) Circ. Res. 61, 301 - 310]. Cardiolipin is normally re-modeled by its de-acylation to mono and di-lysocardiolipin that need to be

transported to the ER for efficient reacylation by acyltransferases. Cardiolipin biosynthesis (Fig 2) is restricted to the inner mitochondria membrane [Hatch GM (1998) *Int. J. Mol. Med.* 1: 33-41]. After the conversion of phosphatidic acid (PA) plus CTP to CDP-diacylglycerol (DAG) and pyrophosphate by CDP-DAG synthase, cardiolipin biosynthesis in eukaryotes occurs in a three-step process. First, phosphatidylglycerophosphate (PGP) synthase catalyzes the formation of PGP from CDP-DAG and 5 $\alpha$ -glycerol-3-phosphate. In the second step, PGP is dephosphorylated to phosphatidylglycerol (PG) by PGP phosphatase. Lastly, cardiolipin synthase catalyzes a phosphatidyl transfer from CDP-DAG to PG, an irreversible reaction that involves cleavage of a high energy anhydride bond to form cardiolipin. Interestingly, BID preferentially interacts with negatively-charged phospholipid phosphatidylglycerol, a precursor of cardiolipin synthesis [Hatch GM (1998) *Int. J. Mol. Med.* 1:33-41]. This suggests that Bid also may be involved in synthesis, or recycling, of cardiolipin.

**[0012]** The regulation of cardiolipin synthesis is important for mitochondrial function in the life cycle of the mammalian cell. Cytochrome c (a proapoptotic factor that binds preferentially to cardiolipin but not to cardiolipin hydroperoxide) associates strongly with cardiolipin [Demel, R. A., Jordi, W., Lambrechts, H., van Damme, H., Hovius, R. and de Kruijff, B. (1989) *J. Biol. Chem.* 264, 3988 - 3997.]. Ostrander et al [Ostrander, D.B., Sparagna, G.C., Amoscato, A.A., McMillin, J.B., and Dowhan, W. (2001) *J. Biol. Chem.* 276 (41), 38061 - 38067] demonstrated that cardiolipin synthesis is directly correlated with release of cytochrome c. Reactive oxygen species (ROS) generated during mitochondrial respiration might be expected to induce the peroxidation of cardiolipin because cardiolipin in mitochondria contains significant quantities of highly unsaturated fatty acids. In a recent study it was observed that peroxidation of cardiolipin in the mitochondria resulted in the

dissociation of cytochrome c from mitochondrial inner membranes, the initial step in the release of cytochrome c from mitochondria [Vik, S. B., Georgevich, G. and Capaldi, R. A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 1456 - 1460.; Ostrander DB et al (2001) *J. Biol. Chem.* 276: 38061 - 38067; Sprang H et al (2001) *Nat. Rev. Mol. Cell. Biol.* 2: 504 - 513]. The quantitative interaction of cytochrome c with anionic mitochondrial phospholipids may predetermine the relative distribution of this protein between mitochondrial membranes and the cytochrome c in energy production and programmed cell death. Evidence has accumulated that cardiolipin plays an integral role as an upstream effectors of these important processes [Sparagna, G. C., Hickson-Bick, D. L., Buja, L. M., and McMillin, J. B. (2000) *Am. J. Physiol.* 279, H2124 - H2132; Watts, J. D., Gu, M., Patterson, S. D., Aebersold, R. and Polverino, A. J. (1999) *Cell Death Differ.* 6, 105 - 114; Listenberger, L. L., Ory, D. S., and Schaffer, J. E. (2001) *J. Biol. Chem.* 276, 14890 - 14895].

[0013] Without being bound by any particular theory, it is believed that inhibition of cardiolipin synthesis in accordance with the inventive method may induce apoptosis by interfering with the ability of BID to target mitochondria [see Lutter, M., Fang M., Luo, X., Nishijima, M., Xiao-song, X., and Wang, X. (2000) *Nature Cell Biology*, 2, 754-756; Listenberger, L. L., Ory, D. S., and Schaffer, J. E. (2001) *J. Biol. Chem.* 276, 14890 - 14895], thus impairing the lipid transfer between the ER and mitochondria. Moreover, the biosynthesis of cardiolipin has been found to be critically affected in a model of lipid-induced apoptosis [Kajiyama, K., Pauly, D. F., Hughes, H., Yoon, S. B., Entman, M. L., and McMillin-Wood, J. B. (1987) *Circ. Res.* 61, 301-310], consistent with the decrease of mitochondrial cardiolipin content during apoptosis induced by various stimuli [Nomura K et al (2000) *Biochem. J.* 351: 183 - 193]. Indeed, several recent studies have probed a link



between cardiolipin levels and apoptosis. For example, in staurosporine-treated granulose cells undergoing apoptosis, cardiolipin levels were observed to be reduced [Khan SM, Dauffenbach LM, Yeh J. (2000) *Biochem Biophys Res Comm*, 269:542-545].

Peroxidation of cardiolipin induced release of cytochrome c from mitochondria into the cytosol and this was associated with the induction of apoptosis [Shidoji Y, Hayashi K, Komura S, Ohishi N, Yagi K. (1999) *Biochem Biophys Res Comm*, 264:343-347; Ushmorov A, Ratter F, Lehmann V, Droge W, Schirmacher V, Umansky V. (1999). *Blood* 1999, 93:2342-2352; Poot M, Pierce RH: (1999) *Cytometry*, 35:311-317].

Suppression of cardiolipin peroxidation also inhibits release of cytochrome c from mitochondria [Nomura K, Imai H, Koumura T, Kobayashi T, Nakagawa Y (2000) *Biochem J*, 351:183-193].

[0014] In accordance with the inventive method, the cell is exposed to an inhibitor of cardiolipin synthesis. Any agent able to inhibit the production of cardiolipin can be employed in the context of the present invention. For example, several compounds that impact cardiolipin synthesis are known in the art, many of which can be suitably used in the context of the inventive method. One exemplary compound is l-Decanoyl-*sn*-glycero-3-phosphorylcholine, (see Schlame et al., *J Biol Chem* 1993 Jan 5; 268 (1): 74-9). Another compound for use in the inventive method is l-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine (Cabener et al., *Br J Pharmacol* 1999 Jun; 127 (4): 813-25). Another compound for use in the inventive method is Hexadecylphosphocholine (Wieder et al., *J Biol Chem* 1998 May 1; 273 (18): 11025-31). Yet another compound that can be used in the inventive method is Lysophosphatidic acid, (Gueguen et al., *Biochem J*. (2002) 368, 447-459). Palmitate is known to diminish the content of mitochondrial synthesis of cardiolipin (Ostrand et al., *J Biol Chem* 2001 Oct; 276 (41) 38061-7) and it can be used

as the inhibitor of cardiolipin in the context of the inventive method. Yet another suitable compound for use in the inventive method is N-(4-hydroxyphenyl)retinamide, which induces oxidation of cardiolipin and leakage of mitochondria and can cause gradual decrease in mitochondrial oxidative turnover and cardiolipin level (Foot et al., Exp. Cell Res. (2002), 10, 279(1), 128-140). Phosphatidyl-3,4-dihydroxybutyl-1-phosphate, which is an analog of glycerol-3-phosphate (Lacombe et al., Biochim Biophys Acta 1989 Sep 25; 1005(2): 103-8), also can be used as the inhibitor of cardiolipin synthesis in the context of the inventive method. Yet another compound that can be used in the context of the inventive method is phosphatidylserine (see Uchida et al, J Biochem (Tokyo) 1998 Jun; 123 (6): 1073-8). Sphingosine-1-phosphate (Endocrinology 2002 Dec; 143 (12): 4755-63) also can be used as the inhibitor in the context of the inventive method). Another compound that can be used as the inhibitor of cardiolipin synthesis is sulfoquinovosyldiacylglycerol (Quasney et al., J Nut Biochem 12 (2001) 310-315). Preferred compounds include l-Decanoyl- $\gamma$ -glycero-3-phosphorylcholine, Lysophosphatidic acid, and Phosphatidyl-3,4-dihydroxy butyl-1-phosphate.

**[0015]** For use *in vivo*, the dosage of any of the foregoing compounds will depend on its manner of formulation and administration. However, optimizing the dosage of such compounds to suit a particular application of the inventive method is within the ordinary skill of the art. Generally, however, the dosage of such compounds for intravenous administration can be as little as about 0.1  $\mu\text{g/kg}$ , such as little as about 0.5  $\mu\text{g/kg}$ , or as little as about 1  $\mu\text{g/kg}$ , and more typically as little as about 2  $\mu\text{g/kg}$  or as little as about 5  $\mu\text{g/kg}$  or even as little as about 10  $\mu\text{g/kg}$ , such as as little as about 25  $\mu\text{g/kg}$  or about 50  $\mu\text{g/kg}$  or about 100  $\mu\text{g/kg}$ . Some compounds can be administered in dosages as little as about 250  $\mu\text{g/kg}$  or as little as about 500  $\mu\text{g/kg}$ . It may be desirable to administer one or

more of the compounds in dosages as little as about 1 mg/kg, such as little as about 2 mg/kg or as little as about 5 mg/kg or 10 mg/kg. Some compounds can be administered in dosages of as little as about 10 mg/kg, such as, as little as about 25 mg/kg or about 50 mg/kg or about 100 mg/kg. Some compounds can be administered in dosages as little as about 250 mg/kg or as little as about 500 mg/kg. The maximum tolerated dose of such compounds can be ascertained by those of skill in the art. Moreover, for intravenous injection, the dosage can be administered continuously for several days or several hours (e.g., about 12 hours or about one or a few hours). For bolus administration, such compounds may be effectively provided to a patient in bolus injections of a few minutes or less, preferably less than one or two minutes, and even more preferably less than about 30 seconds, such as less than about 20 seconds or even less than about 10 or about 5 seconds. Dosages for bolus injections can range from as little as 0.1  $\mu$ g or  $\mu$ g less, or as little as about 0.5  $\mu$ g-1  $\mu$ g, such as as little as about 2 ( $\mu$ g or as little as about 5  $\mu$ g or 10  $\mu$ g, or even as little as about 25  $\mu$ g or 50  $\mu$ g or 100  $\mu$ g. Some compounds can be administered as a bolus in dosages as little as about 250  $\mu$ g or as little as about 500  $\mu$ g. It may be desirable to administer one or more of the compounds as a bolus in dosages as little as about 1 mg, such as little as about 2 mg or as little as about 5 mg or 10 mg. Some compounds can be administered via bolus injection in dosages of as little as about 10 mg, such as, as little as about 25 mg or about 50 mg or about 100 mg. For bolus injection some compounds can be administered in dosages as little as about 250 mg or as little as about 500 mg. Higher dosages are possible, in some applications, and the optimal dosage can be determined by a skilled artisan without the use of undue experimentation.

[0016] In another embodiment, cardiolipin synthesis can be inhibited via recombinant DMA technology, such as via antisense inactivation of the production of one or more

enzymes in the cardiolipin synthesis pathway (see Fig. 2). Antisense inhibition can be achieved using a polynucleotide (e.g., an oligonucleotide) having a sequence consisting essentially of at least a portion of a gene encoding an enzyme involved in the synthesis of cardiolipin. Of course, more than one antisense polynucleotide can be used in concert to achieve redundant expression of the same gene, or to target more than one of the genes involved in the cardiolipin synthetic pathway.

[0017] The portion of the desired gene to which the polynucleotide is antisense can be a coding sequence or a regulatory sequence, such as a 5' or 3' untranslated region, an intron, an exon, a region including a start site or a transcription or translation termination site. Moreover, while the antisense polynucleotide need not be an exact complement of the region of the gene, it should be able to bind to the gene RNA sequence within the cell to attenuate or inhibit expression of the gene encoding the inhibitor of cardiolipin synthesis. Thus, while an exact complement is not required, typically the antisense polynucleotide is an exact complement of at least a portion of a gene encoding an enzyme involved in the synthesis of cardiolipin. While the design of antisense polynucleotides is within the ordinary skill in the art, generally, the antisense polynucleotide will contain at least about 8, and more preferably at least about 12 nucleotides, such as at least about 15 or at least about 20 nucleotides. Antisense polynucleotides containing as many as about 25 or as many as about 30 nucleotides also can be employed, and, indeed, the antisense polynucleotide can contain a larger number of nucleotides, if desired, such as about 40 or about 50 or more nucleotide bases. Generally, however, the antisense polynucleotide contains between about 10 and about 50 nucleotides (such as between about 15 and about 40 nucleotides), while longer or shorter polynucleotides (even substantially longer or shorter) can be employed.

[0018] It is within the ordinary skill of the art to design and produce polynucleotides to achieve antisense inhibition of target genes. Moreover, the genetic sequences of the enzymes catalyzing the synthesis of cardiolipin (e.g., phosphatidylglycerophosphate synthase, phosphatidylglycerophosphate phosphatase, phosphatidate cytidyltransferase 2, cardiolipin synthase, and BID) are known (see, for example, NCBI Entrez Accession No. BC025751 (SEQ ID NO:1), NM\_024419 (SEQ ID NO:2), U75506 (SEQ ID NO:3), NMJ38578 (SEQ ID NO:4), NC\_004741 (SEQ ID NO:5), and AP004603 (SEQ ID NO:6), which are the sequences published by NCBI as of June 23, 2003. Thus, exemplary antisense polynucleotides have sequences consisting of 12-40 base pairs complementary to any of these published sequences. Using these known sequences of these published sequences, an antisense polynucleotide can be derived and constructed using any desired methodology, such as rtPCR of a cDNA library using primers flanking the desired sequence, or using automated oligodeoxyribonucleotide synthesis machines.

[0019] To achieve antisense inhibition, the antisense polynucleotide is introduced into the desired cells such that it is able to interact with the desired RNA target within the cells (e.g., the gene encoding the enzyme involved in the synthesis of cardiolipin). Thus, the antisense sequence can be introduced into the cells directly as "naked" DNA polynucleotides that can be taken up into the cells. Alternatively, the antisense sequence can be engineered into a genetic vector, such as a plasmid or viral vector (e.g., adenoviral, vaccinia viral, herpesviral, retroviral or other suitable viral vector), for efficient transfection or infection of the target cells. In this respect, the antisense polynucleotide can be produced within the cells by engineering the desired antisense sequence into an expression vector operably linked to a promoter for expression within the cells. Once introduced into the cells, the antisense oligonucleotide attenuates or inhibits the

production of cardiolipin within the cells, thus promoting apoptosis.

[0020] The inventive method can be employed on a cell, tissue, or organ explant in vitro, or *in vivo*. The cell type can be of any desired type, such as tissue culture cells, cancer cells, adipose cells, and vascular smooth muscle and endothelial cells.

[0021] When used in vitro, the method can serve as an investigative tool to study apoptosis and the regulation thereof in tissue culture cells, organ explants, etc. In this regard, for in vitro application, cultured cells, explanted tissues containing cells, artificial tissues or organ components, or even explanted organs can be bathed in tissue culture media containing one or more inhibitors of cardiolipin synthesis under conditions permitting the inhibitor of cardiolipin synthesis to contact the cells in question, for example, as discussed above. Vascular tissues, or structures (e.g., organ explants, tissue explants, or artificial organs or tissues) containing vascular vessels or other cavities can alternatively be perfused with a suitable medium containing an inhibitor of cardiolipin synthesis. Thus employed, the method can be used to probe the dosing, time course, and other parameters affecting the inhibition of cardiolipin synthesis within cells. Alternatively, cells, tissues, organs or other structures treated in accordance with the inventive method in vitro can, in some applications, be implanted into a host for therapeutic applications.

[0022] The inventive method also can be used *in vivo* for therapeutic treatment of disorders within human or animal patients. In one embodiment, the inventive method can be employed *in vivo* against adipose tissue, and the "cell" or cells to be treated in accordance with the inventive method can be adipose cell(s). Alternatively, the cell within the adipose tissue can be a connective tissue cell, cells in the stromal-vascular portion of the adipose tissue, or other cells within the adipose tissue. Where employed against

adipose tissue, the inventive method can be used to attenuate the progression of obesity in a patient suffering from obesity. In this regard, the inhibitor of cardiolipin synthesis can cause apoptosis within the adipose tissue (e.g., of adipose cells, connective tissue, and/or stromal or other cells) and/or inhibit the proliferation or growth of such cells within the patient, which can reduce the volume of (or at least retard the growth of) adipose tissue within the patient. In this regard, the inventive method can attenuate the progression of obesity (e.g., adipose growths) within the patient. As with the treatment of cancers and tumors noted above, a preferred method for introducing the inhibitor of cardiolipin synthesis within adipose tissue is via direct inter-tissue (e.g., interstitial) injection, such as via convection-enhanced delivery.

[0023] In attenuating the progression of obesity within the patient, the inventive method need not achieve reduction in obesity, although this is preferred. Indeed, it is often sufficient for the inventive method to reduce the progression of the disease within the patient. However, it is desirable for the inventive method to achieve remission of the disorder or even to reverse obesity in some patients, e.g., leading to a reduction in adipose tissue volume or mass, and a loss of weight for the patient. Moreover, it will be understood that the inventive method can be used in conjunction with, or adjunctively with, drugs or pharmaceutical agents that also treat obesity. Similarly, the inventive method can be used in conjunction with surgical procedures, dietary and behavior modification therapy, and other strategies for treating obesity within afflicted patients.

[0024] In another embodiment, the inventive method can be directed against cells of the cardiovascular system, for example, vascular smooth muscle cells and endothelial cells. Such cells typically are within the lumens of the vasculature (e.g., arterial lumens, venous lumens, etc.). Thus employed, the inventive method can be used to treat a patient

suffering from a cardiovascular disease. Exemplary cardiovascular diseases that can be treated in accordance with the inventive method include those characterized by the buildup of fatty plaque deposits in vascular walls. In accordance with the inventive method, the inhibitor of cardiolipin synthesis (e.g., within a suitable composition also including a pharmaceutically-acceptable carrier) is administered to the patient under conditions sufficient to inhibit proliferation of fatty plaque deposits in vascular walls. For example, by inducing apoptosis within such cells, the inventive method can retard the proliferation of these cells within the vascular tissue. While it is sufficient for the inventive method to attenuate the proliferation of such cells, in some embodiments, the inventive method can halt the proliferation of such cells, and thereby block the continued build-up of fatty plaque within the vessel lumen. It is more preferred for the inventive method to reduce the number of such cells, and thereby achieve a reduction in the amount of plaque present within the vascular tissue.

[0025] For treatment of cardiovascular diseases, typically the composition including the inhibitor of cardiolipin synthesis is delivered to the patient in situ within a desired site of a blood vessel. For in situ delivery of a vector internally, the region of interest desirably is further segregated from the remainder of the patient's tissue. Any of a variety of known surgical procedures for physically segregating the region of interest is appropriate. Various endovascular surgical techniques appropriate for segregating a region of interest are available, depending upon the location of the target. Endovascular surgical procedures include, but are not limited to, balloon angioplasty, intravascular stents, laser-assisted balloon angioplasty, double balloon catheterization, mechanical endarterectomy and vascular endoscopy. For a review of endovascular alternatives, see generally Ahn, "Endovascular Surgery," in *Vascular Surgery, A Comprehensive Review*, Ed. W. S.



Moore, W. B. Saunders & Co., Philadelphia (1993)).

[0026] Several catheter designs can be utilized for local delivery of a composition including an inhibitor of cardioliipin synthesis to the patient. One catheter design consists of two independently inflated balloons, one proximal and one distal to the vascular delivery site. Inflation of these balloons provides an evacuated isolated arterial segment into which a composition including an inhibitor of cardioliipin synthesis can be infused. This system is, however, limited by a failure to provide distal arterial perfusion. A second catheter design developed by Wolinsky allows the infusion of the composition including an inhibitor of cardioliipin synthesis through 25-100  $\mu\text{M}$  pores under pressures up to 5 atm. This perfusion pressure increases the depth of penetration by the composition including an inhibitor of cardioliipin synthesis and, where the inhibitor is a genetic vector, can additionally increase the transfer efficiency of the vector into the cells. Yet another catheter design utilizes an expandable stent, which traps the balloon against the arterial wall and allows intramural delivery of the composition including an inhibitor of cardioliipin synthesis through spaces in the stent material. Additionally, these stents can be modified with burrs, which create holes deeper in the vessel wall and allow flow of the composition including an inhibitor of cardioliipin synthesis to these sites to allow more uniform delivery throughout the vessel wall. Also, biodegradable stents formed from agents such as an ethylenevinyl acetic copolymer are appropriate for localized delivery to vascular tissue. Alternatively, an intravascular stent can be utilized wherein the endovascular scaffold of the stent is bathed in a ointment, cream, lotion, colloidal dispersion such as a gel or magma or any other acceptable carrier which comprises the inhibitor of cardioliipin synthesis for delivery to the targeted portion of a vessel segment. This solution is applicable to either an in situ or ex vivo based vessel delivery. Another

specific application, offered for the purpose of example and not of limitation, is the use of a self-expanding stent. This intravascular stent can be bathed in a gel solution comprising an inhibitor of cardiolipin synthesis and delivered percutaneously to the target vessel site. An initial angioplasty, if necessary, is followed by delivery of the bathed scaffold to the target vessel site. The delivery catheter is removed and the scaffold is dilated with a conventional balloon. It is within the purview of the skilled vascular surgeon to use other types of intravascular stents such as a balloon expandable stent or a thermal expanding stent. Additionally, numerous balloon catheters of varying sizes, shapes, and types are available to the skilled vascular surgeon for endovascular delivery of the composition including an inhibitor of cardiolipin synthesis.

[0027] The inventive method, of course, can be employed in connection with surgical endovascular techniques, such as procedures to bypass a vascular occlusion. Such procedures typically involve a homograft or heterograft comprising an artery or vein, or a segment thereof, or an artificial conduit. Vascular bypass procedures involve forming a proximal and distal anastomosis between the graft conduit and the vessel. A composition including an inhibitor of cardiolipin synthesis then can be transferred to the cells in the region of the anastomoses to promote proper healing of the surgical wound between the two conduits. Where the graft conduit is not artificial (e.g., an artery, a vein, or a segment thereof), the composition including an inhibitor of cardiolipin synthesis can be transferred to the cells of the graft lumen. Additional preferred methods for delivering a composition including an inhibitor of cardiolipin synthesis to a vessel *in vivo* or *ex vivo* involve vascular surgery.

[0028] In yet another embodiment, the cell treated in accordance with the inventive method is a cancerous cell. For example, the cell to be treated in accordance with the

inventive method can be selected from the group of cancer cells consisting of lung cancer, bronchus cancer, colorectal cancer, prostate cancer, breast cancer, pancreas cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, esophageal cancer, cervical cancer, melanoma, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, liposarcoma, testes cancer, lymphoma, multiple myeloma, and leukemia. Of course, other types of cancer cells also can be treated in accordance with the inventive method.

[0029] When employed *in vivo* against cancer cells, the invention affords a method of attenuating the progression of a cancer in a patient suffering from cancer by administering to the patient an inhibitor of cardiolipin synthesis under conditions sufficient to inhibit progression of said cancer within said patient. For disseminated or metastasized cancer, the conditions can be satisfied by intravenous administration of the inhibitor of cardiolipin synthesis. For topical cancers, such as melanoma or other skin or epithelial cancers, the method can involve topical application of a composition (e.g., a gel, magma, creme, suppository, etc.) containing the inhibitor of cardiolipin synthesis. In many applications, the cancer cell or cells to be treated are within or form a tumor or other similar structure.

[0030] In such instances in which the cancer cell is within a tumor, the invention affords a method of attenuating the growth of the tumor by exposing the tumor to an inhibitor of cardiolipin synthesis under conditions sufficient to attenuate the growth of said tumor. Ideally, the inventive method is used to treat a cancer manifested as a solid tumor or a tumor associated with soft tissue (i.e., soft tissue sarcoma) in a human. The tumor can be associated with cancers of (i.e., located in) the oral cavity and pharynx, the digestive

system, the respiratory system, bones and joints (e.g., bony metastases), soft tissue, the skin (e.g., melanoma), breast, the genital system, the urinary system, the eye and orbit, the brain and nervous system (e.g., glioma), or the endocrine system (e.g., thyroid) and is not necessarily the primary tumor. Tissues associated with the oral cavity include, but are not limited to, the tongue and tissues of the mouth. Cancer can arise in tissues of the digestive system including, for example, the esophagus, stomach, small intestine, colon, rectum, anus, liver, gall bladder, and pancreas. Cancers of the respiratory system can affect the larynx, lung, and bronchus and include, for example, non-small cell lung carcinoma. Tumors can arise in the uterine cervix, uterine corpus, ovary, vulva, vagina, prostate, testis, and penis, which make up the male and female genital systems, and the urinary bladder, kidney, renal pelvis, and ureter, which comprise the urinary system. The target tissue also can be associated with lymphoma (e.g., Hodgkin's disease and Non-Hodgkin's lymphoma), multiple myeloma, or leukemia (e.g., acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia, chronic myeloid leukemia, and the like). The tumor can be at any stage, and can be subject to other therapies. The inventive method is useful in treating tumors that have been proven to be resistant to other forms of cancer therapy, such as radiation-resistant tumors. The tumor also can be of any size. A preferred method of treating cancerous tumors in accordance with the inventive method involves direct intratumoral or interstitial injection of a composition containing the inhibitor of cardiolipin synthesis. One known process for achieving such direct injection is via convection enhanced delivery (see, e.g., U.S. Patent 5,720,720).

[0031] Where the method is employed to attenuate the progression of cancer within a patient, or to attenuate the growth of a tumor in a patient, the method need not achieve complete elimination or remission of the cancer or tumor. In this regard, a successful

therapeutic treatment can include halting the progression of the cancer or tumor, thereby enlarging the time that the growing cancer or tumor can be treated by other methods. In this regard, the inventive method can be employed adjunctively with other methods and reagents for treating cancerous cells and tumor. For example, the method can be employed in conjunction with radiation therapy of cancers or tumors. Alternatively, the inventive method can be used in conjunction with chemotherapeutic methods. Thus, when used to treat cancer cells, the inventive method can include adjunctively exposing the cell or cells to be treated, or a tumor containing them, with one or more antineoplastic agents or other drugs, many of which are known in the art. For example, drugs or active agents for adjunctive use in conjunction with the inventive method can include anticancer agents (e.g., chemotherapeutic agents), in that they are capable of inducing (either directly or indirectly) cancer cell or tumor cell cytotoxicity. Exemplary anticancer agents include mitoxantrone, taxanes, paclitaxel, camptothecin, camptothecin derivatives (e.g., SN-38), topotecan, gemcitabine, vinorelbine, vinblastine, anthracyclines, adriamycin, capecitabine, doctaxel, didanosine (ddl), stavudine (d4T), antisense oligonucleotides (e.g., c-raf antisense oligonucleotide (RafAON)), antibodies (e.g., herceptin), immunotoxins, hydroxyurea, melphalan, chlormethine, extramustinephosphate, uramustine, ifosfamide, mannomustine, trifosfamide, streptozotocin, mitobronitol, mitoxantrone, methotrexate, 5-fluorouracil, cytarabine, tegafur, idoxide, taxol, daunomycin, daunorubicin, bleomycin, amphotericin, carboplatin, cisplatin, BCNU, vincristine, camptothecin, mitomycin, doxorubicin, etoposide, histermine dihydrochloride, tamoxifen, cytoxan, leucovorin, oxaliplatin, irinotecan, raltitrexed, epirubicin, anastrozole, proleukin, sulindac, EKI-569, erthroxylaceae, cerubidine, docetaxel, cytokines (e.g., interleukins), ribozymes, interferons, oligonucleotides, and functional derivatives of the foregoing.

[0032] In a preferred embodiment of the invention, an anticancer agent for adjunctive use with the inhibitor of cardiolipin synthesis can be an antisense oligonucleotide, typically comprising at least between about 7 and 13 nucleotides and up to between about 32 and 38 nucleotides (e.g., between about 10 and about 35 nucleotides) directed against a gene encoding a product that promotes tumor initiation and/or progression. A preferred antisense nucleotide targets c-raf. (e.g., a c-raf antisense oligonucleotide (RafAON)). Where such oligonucleotides are included, the formulation can additionally include at least one drug, such as paclitaxel, mitoxantrone, camptothecins (preferably 7-ethyl-10-hydroxycamptothecin, i.e., SN-38) doxorubicin, gemcitabine, vinorelbine, vinblastine, cisplatin, 5-fluorouracil, mitomycin, and adriamycin. Methods of using certain of the aforementioned drugs in formulations to treat cancer are known in the art and are described in, for example, Pathak et al., *J. Am. Coll. Nutr.*, 21, 416-421 (2002), Socinski et al., *Cancer*, 95, 1520-1527 (2002), Lewis et al., *Cancer Chemother. Pharmacol*, 50, 257-265 (2002), Ricci et al., *Cancer*, 95, 1444-1450 (2002), Park et al., *Breast Cancer Res.*, 4, 95-99 (2002), Thigpen, T., *Semin. Oncol*, 29 (1 Suppl. 1), 11-16 (2002), and U.S. Patent 5,744,460, all of which are incorporated herein by reference.

[0033] Other drugs or active agents which can be employed adjunctionally in the inventive method include agents which act on the peripheral nerves, adrenergic receptors, cholinergic receptors, the skeletal muscles, the cardiovascular system, smooth muscles, the blood circulatory system, synaptic sites, neuroeffector junctional sites, endocrine and hormone systems, the immunological system, the reproductive system, the skeletal system, the alimentary and excretory systems, the histamine system and the central nervous system. Suitable agents may be selected from, for example, proteins, enzymes, hormones, nucleotides, polynucleotides, nucleoproteins, polysaccharides, glycoproteins, lipoproteins,

polypeptides, steroids, terpenoids, retinoids, anti-ulcer H2 receptor antagonists, antiulcer drugs, hypocalcemic agents, moisturizers, cosmetics, etc. Active agents can be analgesics; anesthetics; anti-arrhythmic agents, antibiotics; antiallergic agents, antifungal agents, antihypertensive agents (e.g., dihydropyridines, antidepressants, cox-2 inhibitors); anticoagulants; antidepressants; antidiabetic agents, anti-epilepsy agents, antiinflammatory corticosteroids; agents for treating Alzheimers or Parkinson's disease; antiulcer agents; anti-protozoal agents, anxiolytics, thyroids, anti-thyroids, antivirals, anoretics, bisphosphonates, cardiac inotropic agents, cardiovascular agents, corticosteroids, diuretics, dopaminergic agents, gastrointestinal agents, hemostatics, hypercholesterol agents, antihypertensive agents; immunosuppressive agents; anti-gout agents, anti-malarials, anti-migraine agents, antimuscarinic agents, antiinflammatory agents, such as agents for treating rheumatology, arthritis, psoriasis, inflammatory bowel disease, Crohn's disease; or agents for treating demyelinating diseases including multiple sclerosis; ophthalmic agents; vaccines (e.g., against influenza virus, pneumonia, hepatitis A, hepatitis B, hepatitis C, cholera toxin B-subunit, typhoid, plasmodium falciparum, diphtheria, tetanus, herpes simplex virus, tuberculosis, HIV, bordetella pertussis, measles, mumps, rubella, bacterial toxoids, vaccinia virus, adenovirus, canary virus, bacillus calmette Guerin, klebsiella pneumonia vaccine, etc.); histamine receptor antagonists, hypnotics, kidney protective agents, lipid regulating agents, muscle relaxants, neuroleptics, neurotropic agents, opioid agonists and antagonists, parasympathomimetics, protease inhibitors, prostaglandins, sedatives, sex hormones (e.g., androgens, estrogens, etc.), stimulants, sympathomimetics, vasodilators, xanthins, and synthetic analogs of these species.

[0034] The agents or drugs used adjunctively in connection with the inventive method can be nephrotoxic, such as cyclosporins and amphotericin B, or cardiotoxic, such as

amphotericin B and paclitaxel. Additional examples of drugs which may be delivered by way of the inventive composition include, prochlorperazine edisylate, ferrous sulfate, aminocaproic acid, mecamlamine hydrochloride, procainamide hydrochloride, amphetamine sulfate, methamphetamine hydrochloride, benzamphetamine hydrochloride, isoproterenol sulfate, phenmetrazine hydrochloride, bethanechol chloride, methacholine chloride, pilocarpine hydrochloride, atropine sulfate, scopolamine bromide, isopropamide iodide, tridihexethyl chloride, phenformin hydrochloride, methylphenidate hydrochloride, theophylline choline, cephalexin hydrochloride, diphenidol, meclizine hydrochloride, prochlorperazine maleate, phenoxybenzamine, thiethylperazine maleate, anisidone, diphenadione erythrityl tetranitrate, digoxin, isoflurophate, acetazolamide, methazolamide, bendroflumethiazide, chloropromaide, tolazamide, chlormadinone acetate, phenaglycodol, allopurinol, aluminum aspirin, methotrexate, acetyl sulfisoxazole, erythromycin, hydrocortisone, hydrocorticosterone acetate, cortisone acetate, dexamemasone and its derivatives such as betamethasone, triamcinolone, methyltestosterone, 17-S-estradiol, ethinyl estradiol, ethinyl estradiol 3-methyl ether, prednisolone, 17a-hydroxyprogesterone acetate, 19-norprogesterone, norgestrel, norethindrone, norethisterone, norethiederone, progesterone, norgesterone, norethynodrel, aspirin, indomethacin, naproxen, fenoprofen, indoprofen, nitroglycerin, isosorbide dinitrate, propranolol, timolol, atenolol, alprenolol, cimetidine, clonidine, imipramine, levodopa, chlorpromazine, methyl dopa, dihydroxyphenylalanine, theophylline, calcium gluconate, ketoprofen, ibuprofen, cephalexin, haloperidol, zomepirac, ferrous lactate, vincamine, diazepam, phenoxybenzamine, diltiazem, milrinone, mandol, quanbenz, hydrochlorothiazide, ranitidine, flurbiprofen, fenufen, fluprofen, tolmetin, alclofenac, mefenamic, flufenamic, difuinal, niraodipine, nitrendipine, nisoldipine, nicardipine, felodipine, lidoflazine,



tiapamil, gallopamil, amlodipine, mioflazine, lisinopril, enalapril, enalaprilat captopril, ramipril, famotidine, nizatidine, sucralfate, etintidine, tetratolol, minoxidil, chlordiazepoxide, diazepam, amitriptyline, and imipramine. Further examples are proteins and peptides which include, but are not limited to, bone morphogenic proteins, insulin, heparin, colchicine, glucagon, thyroid stimulating hormone, parathyroid and pituitary hormones, calcitonin, renin, prolactin, corticotrophin, thyrotropic hormone, follicle stimulating hormone, chorionic gonadotropin, gonadotropin releasing hormone, somatotropins (e.g., bovine somatotropin, porcine somatotropin, etc.), oxytocin, vasopressin, GRF, somatostatin, lypressin, pancreozymin, luteinizing hormone, LHRH, LHRH agonists and antagonists, leuprolide, interferons (e.g.,  $\alpha$ -,  $\beta$ -, or  $\gamma$ -interferon, interferon  $\alpha$ -2a, interferon  $\alpha$ -2b, and consensus interferon, etc.), interleukins, growth hormones (e.g., human growth hormone and its derivatives such as methionine-human growth hormone and des-phenylalanine human growth hormone, bovine growth hormone, porcine growth hormone, insulin-like growth hormone, etc.), fertility inhibitors such as the prostaglandins, fertility promoters, growth factors such as insulin-like growth factor, coagulation factors, pancreas hormone releasing factor, analogs and derivatives of these compounds, and pharmaceutically acceptable salts of these compounds, or their analogs or derivatives.

[0035] In the context of the inventive method, a therapeutically effective amount of the inhibitor of cardiolipin synthesis (and any additional adjunctive agent) is administered to a mammalian host, most preferably a human host, to treat a condition, such as cancer, obesity, or cardiovascular disease. A "therapeutically effective amount" means an amount sufficient to show a meaningful benefit in an individual, i.e., promoting at least one aspect of apoptosis, tumor cell cytotoxicity, or treatment, healing, prevention, or amelioration of

other relevant medical condition(s) associated with a particular disorder. Therapeutically effective amounts may vary depending upon the biological effect desired in the individual, disorder to be treated, and/or the specific characteristics of the inhibitor of cardiolipin synthesis (and any additional adjunctive agent), and individual. Thus, the attending physician (or other medical professional responsible for administering the composition) will typically decide the amount of inhibitor of cardiolipin synthesis (and any additional adjunctive agent) with which to treat each individual patient.

[0036] The inhibitor of cardiolipin synthesis (and any additional adjunctive agent) preferably is included in a pharmaceutical preparation in dosage units. This means that the preparations are in the form of individual parts, for example capsules, pills, suppositories and ampoules, of which the content of the liposome composition corresponds to a fraction or a multiple of an individual dose. The dosage units can contain, for example, 1, 2, 3 or 4 individual doses or a fraction of (e.g., 1/2, 1/3, or 1/4, etc.) of an individual dose. An individual dose preferably contains the amount of the liposome which is given in one administration and which usually corresponds to a whole, a half, a third, or a quarter of a daily dose. In this regard, the liposome should preferably be present in a pharmaceutical preparation at a concentration of about 0.01 to 5 wt.%, about 0.05 to 1 wt.%, about 0.1 to 1.5 wt.%, about 0.2 to 1 wt.%, or about 0.5 to 1 wt.% relative to the total mixture. However, it can be necessary to deviate from the dosages mentioned and in particular to do so as a function of the nature and body weight of the subject to be treated, the nature and the severity of the illness, the nature of the preparation and if the administration of the medicine, and the time or interval over which the administration takes place. Thus it can suffice in some cases to manage with less than the abovementioned amount of active compound, whilst in other cases the abovementioned amount of active compound must be

exceeded. The particular required optimum dosage and the type of administration of the inhibitor of cardiolipin synthesis (and any additional adjunctive agent) can be determined by one skilled in the art, by available methods. Suitable amounts are therapeutically effective amounts that do not have excessive toxicity, as determined in empirical studies.

[0037] In accordance with the inventive method, the inhibitor of cardiolipin synthesis (and any additional adjunctive agent) desirably is formulated into a pharmaceutical composition comprising a physiologically acceptable (e.g., a pharmaceutically or pharmacologically acceptable) carrier (e.g., excipient or diluent). Any suitable physiologically acceptable carrier can be used within the context of the invention, and such carriers are well known in the art. Most preferably, the inventive method employs a non-toxic, inert physiologically-acceptable carrier. Such carriers are known in the art and include, for example, semi-solid or liquid diluents, fillers and formulation auxiliaries of all kinds. The carrier typically will be liquid, but also can be solid, or a combination of liquid and solid components. The choice of carrier will be determined, at least in part, by the location of the target tissue and/or cells, and the particular method used to administer the composition.

[0038] Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared, and the preparations can also be emulsified. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions, formulations including sesame oil, peanut oil or aqueous propylene glycol, and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of

manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxycellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0039] The inhibitor of cardiolipin synthesis (and other adjunctive agents) for use in the present invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such as organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups also can be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. The composition can further comprise any other suitable components, especially for enhancing the stability of the composition and/or its end-use. Accordingly, there is a wide variety of suitable formulations of the composition of the invention. The following formulations and methods are merely exemplary and are in no way limiting. Formulations in accordance with these exemplary types can be manufactured in the usual manner according to known methods, for example by mixing the inhibitor of cardiolipin synthesis (and any other adjunctive active agents) with the appropriate excipient or excipients.

[0040] For oral administration, the inhibitor of cardiolipin synthesis (and other adjunctive agents) can be formulated as tablets, capsules, lozenges, powders, syrups, aqueous solutions, suspensions, and the like. Carriers such as lactose, sodium citrate, and salts of phosphoric acid can be used to prepare tablets. Further, disintegrants such as starch, and lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc can be included. Diluents such as lactose and high molecular weight polyethylene glycols can be used in the preparation of dosages in capsule form. The active ingredient can be combined with emulsifying and suspending agents to generate aqueous suspensions for oral use. Flavoring agents such as sweeteners can be added, as desired.

[0041] For topical (i.e., dermal) administration, the inhibitor of cardiolipin synthesis (and other adjunctive agents) can be provided in the form of gels, oils, and emulsions by the addition of suitable water-soluble or water-insoluble excipients, for example polyethylene glycols, certain fats, and esters or mixtures of these substances. Suitable excipients are those in which the liposome composition is sufficiently stable to allow for therapeutic use.

[0042] Formulations suitable for anal administration can be prepared as suppositories by mixing the inhibitor of cardiolipin synthesis (and other adjunctive agents) with a variety of bases such as emulsifying bases or water-soluble bases. Formulations suitable for vaginal administration can be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

[0043] Formulations suitable for administration of the inhibitor of cardiolipin synthesis (and other adjunctive agents) via inhalation include aerosol formulations. The aerosol formulations can be placed into pressurized acceptable propellants, such as

dichlorodifluoromethane, propane, nitrogen, and the like. They also can be formulated as non-pressurized preparations, for delivery from a nebulizer or an atomizer.

[0044] Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. In a preferred embodiment of the invention, the liposome composition is formulated for injection. In this regard, the formulation desirably is suitable for intratumoral administration, but also can be formulated for intravenous injection, intraperitoneal injection, subcutaneous injection, and the like. In this manner, for example, liposome formulations containing two or more anticancer drugs may be injected directly into tumor tissue for delivery of the anticancer drugs directly to cancer cells. In some cases, particularly after resection of a tumor, the liposome formulation can be implanted directly into the resulting cavity or may be applied to the remaining tissue as a coating. In cases in which the liposome formulation is administered after surgery, it is possible to utilize liposomes having larger diameters of about 1 micron since they do not have to pass through the vasculature.

[0045] In addition to the inhibitor of cardioliipin synthesis (and other adjunctive agents), the composition can comprise additional therapeutic or biologically-active agents. For example, therapeutic factors (e.g., antibodies) useful in the treatment of a particular indication can be present. Factors that control inflammation, such as ibuprofen or steroids, can be part of the composition to reduce swelling and inflammation associated with *in vivo* administration of the inhibitor of cardioliipin synthesis (and other adjunctive agents) and physiological distress. Immune system suppressors can be administered with the composition to reduce any immune response to the antibody itself or associated with a disorder. Alternatively, immune enhancers can be included in the composition to up-regulate the body's natural defenses against disease. Moreover, cytokines can be administered with the composition to attract immune effector cells to a disease (e.g., tumor) site.

[0046] Preferred formulations for use *in vivo* can include liposomes. Accordingly, for use in the inventive method, the invention also provides a pharmaceutical composition including an inhibitor of cardioliipin synthesis (e.g., an antibody, genetic vector, polynucleotide, or small molecule inhibitor of cardioliipin synthesis, such as described above) and a liposome. Desirably, the inhibitor of cardioliipin synthesis is entrapped in the liposome, such as within the lipid fraction or the lumen of the liposomes within the composition.

[0047] Where such liposomal formulations of an antibody, genetic vector, polynucleotide, or small molecule inhibitor of cardioliipin synthesis are employed, it is desirable for the liposomal fraction to contain cardioliipin among the lipids. The cardioliipin can be a natural or a synthetic cardioliipin and it can be neutral, or charged positively or negatively, as desired. The precise formulation of the inhibitor of cardioliipin

synthesis, however, is not critical to the inventive method, and it is within the ordinary skill of the art to formulate active agents, such as antibodies, genetic vectors, antisense polynucleotides, and small molecule "drugs" into such formulations for intravenous injection, or for other modes of application, into liposomal formulations.

[0048] All references, including publications, patent applications, and patents, cited herein, including those cited above in the text of the specification, and in the following list, are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0049] The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.



[0050] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

## WHAT IS CLAIMED IS:

1. A method of inducing apoptosis in a cell, said method comprising exposing the cell to an inhibitor of cardiolipin synthesis under conditions sufficient to induce apoptosis within the cell.
2. The method of claim 1 wherein the cell is *in vivo*.
3. The method of claim 1 or 2, wherein the cell is a cancerous cell.
4. The method of claim 3, wherein the cells is selected from the group of cancer cells consisting of lung cancer, bronchus cancer, colorectal cancer, prostate cancer, breast cancer, pancreas cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, esophageal cancer, cervical cancer, melanoma, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, liposarcoma, testes cancer, lymphoma, multiple myeloma and leukemia.
5. The method of claim 4, wherein the cell is also exposed to an anti-neoplastic agent.
6. The method of claim 5, wherein the antineoplastic agent is selected from the group of antineoplastic agents consisting of mitoxantrone, taxanes, paclitaxel, camptothecin, camptothecin derivatives, topotecan, gemcitabine, vinorelbine, vinblastine, anthracyclines, adriamycin, capecitabine, docetaxel, didanosine (ddl), stavudine (d4T), antisense oligonucleotides, antibodies, immunotoxins, hydroxyurea, melphalan, chlormethine, extramustinephosphate, uramustine, ifosfamide, mannomustine, trifosfamide, streptozotocin, mitobronitol, mitoxantrone, methotrexate, 5-fluorouracil, cytarabine, tegafur, idoxide, taxol, daunomycin, daunorubicin, bleomycin, amphotericin, carboplatin, cisplatin, BCNU,

vincristine, mitomycin, doxorubicin, etoposide, histermine dihydrochloride, tamoxifen, cytoxan, leucovorin, oxaliplatin, irinotecan, raltitrexed, epirubicin, anastrozole, proleukin, sulindac, EKI-569, erthoxylaceae, cerubidine, docetaxel, cytokines, ribozymes, interferons, oligonucleotides, and functional derivatives and combinations thereof.

7. The method of claim 1 or 2, wherein the cell is an adipose cell.
8. The method of claim 1 or 2, wherein the cell is a cardiovascular cell.
9. The method of claim 8, wherein the cell is selected from the group of cardiovascular cells consisting of vascular smooth muscle cells and endothelial cells.
10. A method of attenuating the progression of a cancer in a patient suffering from cancer, said method comprising administering to said patient an inhibitor of cardiolipin synthesis under conditions sufficient to inhibit progression of said cancer within said patient.
11. The method of claim 10, wherein the cancer is selected from the group of cancers consisting of lung cancer, bronchus cancer, colorectal cancer, prostate cancer, breast cancer, pancreas cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, esophageal cancer, cervical cancer, melanoma, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, liposarcoma, testes cancer, lymphoma, multiple myeloma and leukemia.
12. The method of claim 11, wherein the inhibitor of cardiolipin synthesis is delivered to said patient within a pharmaceutical composition comprising said inhibitor of cardiolipin synthesis and a pharmaceutically acceptable carrier.

13. The method of claim 11, wherein said cancer comprises a tumor and wherein said composition is delivered to said patient by direct injection at the site of said tumor.

14. The method of claim 11, wherein the patient is also exposed to an antineoplastic agent.

15. The method of claim 14, wherein the antineoplastic agent is selected from the group of antineoplastic agents consisting of mitoxantrone, taxanes, paclitaxel, camptothecin, camptothecin derivatives, topotecan, gemcitabine, vinorelbine, vinblastine, anthracyclines, adriamycin, capecitabine, doctaxel, didanosine (ddl), stavudine (d4T), antisense oligonucleotides, antibodies, immunotoxins, hydroxyurea, melphalan, chlormethine, extramustinephosphate, uramustine, ifosfamide, mannomustine, trifosfamide, streptozotocin, mitobronitol, mitoxantrone, methotrexate, 5-fluorouracil, cytarabine, tegafur, idoxide, taxol, daunomycin, daunorubicin, bleomycin, amphotericin, carboplatin, cisplatin, BCNU, vincristine, mitomycin, doxorubicin, etoposide, histermine dihydrochloride, tamoxifen, cytoxan, leucovorin, oxaliplatin, irinotecan, raltitrexed, epirubicin, anastrozole, proleukin, sulindac, EKI-569, erthroxyloaceae, cerubidine, docetaxel, cytokines, ribozymes, interferons, oligonucleotides, and functional derivatives, and combinations thereof.

16. A method of attenuating the growth of a tumor, said method comprising exposing the tumor to an inhibitor of cardiolipin synthesis under conditions sufficient to attenuate the growth of said tumor.

17. The method of claim 16, wherein the tumor is *in vivo*.

18. The method of claim 16, wherein the tumor comprises cancerous cells.

19. The method of claim 18, wherein the cancerous cells are selected from the group of cancers consisting of lung cancer, bronchus cancer, colorectal cancer, prostate

cancer, breast cancer, pancreas cancer, stomach cancer, ovarian cancer, urinary bladder cancer, brain or central nervous system cancer, peripheral nervous system cancer, esophageal cancer, cervical cancer, melanoma, uterine or endometrial cancer, cancer of the oral cavity or pharynx, liver cancer, kidney cancer, biliary tract cancer, small bowel or appendix cancer, salivary gland cancer, thyroid cancer, adrenal gland cancer, osteosarcoma, chondrosarcoma, liposarcoma, testes cancer, lymphoma, multiple myeloma, and leukemia.

20. The method of claim 18, wherein the tumor also is exposed to an antineoplastic agent.

21. The method of claim 18, wherein the antineoplastic agent is selected from the group of antineoplastic agents consisting of mitoxantrone, taxanes, paclitaxel, camptothecin, camptothecin derivatives, topotecan, gemcitabine, vinorelbine, vinblastine, anthracyclines, adriamycin, capecitabine, docetaxel, didanosine (ddl), stavudine (d4T), antisense oligonucleotides, antibodies, immunotoxins, hydroxyurea, melphalan, chlormethine, extramustinephosphate, uramustine, ifosfamide, mannomustine, trifosfamide, streptozotocin, mitobronitol, mitoxantrone, methotrexate, 5-fluorouracil, cytarabine, tegafur, idoxide, taxol, daunomycin, daunorubicin, bleomycin, amphotericin, carboplatin, cisplatin, BCNU, vincristine, mitomycin, doxorubicin, etoposide, histemine dihydrochloride, tamoxifen, cytoxan, leucovorin, oxaliplatin, irinotecan, raltitrexed, epirubicin, anastrozole, proleukin, sulindac, EKI-569, erthroxyllaceae, cerubidine, docetaxel, cytokines, ribozymes, interferons, oligonucleotides, and functional derivatives and combinations thereof.

22. A method of attenuating the progression of obesity in a patient suffering from obesity, said method comprising administering to said patient an inhibitor of

cardiolipin synthesis under conditions sufficient to inhibit proliferation or growth of adipose cells within said patient.

23. The method of claim 22, wherein the inhibitor of cardiolipin synthesis is delivered to said patient within a pharmaceutical composition comprising said inhibitor of cardiolipin synthesis and a pharmaceutically acceptable carrier.

24. The method of claim 22, wherein said composition is delivered to said patient by direct injection into adipose tissue.

25. A method of attenuating the progression of an of an adipose growth, said method comprising exposing the adipose growth to an inhibitor of cardiolipin synthesis under conditions sufficient to attenuate the progression of said adipose growth.

26. The method of claim 25, wherein said adipose growth is *in vivo*.

27. A method of treating a patient suffering from a cardiovascular disease characterized by the buildup of fatty plaque deposits in vascular walls, said method comprising administering to said patient an inhibitor of cardiolipin synthesis under conditions sufficient to inhibit proliferation of fatty plaque deposits in vascular walls within said patient.

28. A method of treating a patient suffering from a cardiovascular disease characterized by the buildup of fatty plaque deposits in vascular walls, said method comprising administering to said patient an inhibitor of cardiolipin synthesis under conditions sufficient to reduce the amount of plaque present within the vascular tissue.

29. The method of claim 27 or 28, wherein the inhibitor of cardiolipin synthesis is delivered to said patient within a pharmaceutical composition comprising said inhibitor of cardiolipin synthesis and a pharmaceutically acceptable carrier.

30. The method of any of claims 1 -29, wherein the inhibitor of cardiolipin synthesis is selected from the group of compounds consisting of 1-decanoyl-*sn*-glycero-3-phosphorylcholine, 1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine, hexadecylphosphocholine, lysophosphatidic acid, palmitate, N-(4-hydroxyphenyl)retinamide, phosphatidyl-3,4-dihydroxybutyl-1-phosphate, phosphatidylserine, sphingosine-1-phosphate, and sulfoquinovosyldiacylglycerol.

31. The method of any of claims 1-29, wherein the inhibitor of cardiolipin synthesis is a polynucleotide having a sequence antisense to the coding sequence of an enzyme in the cardiolipin synthesis pathway.

32. The method of any of claims 1-29, wherein the inhibitor of cardiolipin synthesis is a polynucleotide having a sequence antisense to the regulatory sequence of an enzyme in the cardiolipin synthesis pathway.

33. The method of claim 31 or 32, wherein the enzyme is selected from the group of enzymes consisting of phosphatidylglycerophosphate synthase, phosphatidylglycerophosphate phosphatase and cardiolipin synthase.

34. A pharmaceutical composition comprising an inhibitor of cardiolipin synthesis and a liposomal carrier.

35. The composition of claim 34, wherein the inhibitor of cardiolipin synthesis is selected from the group of compounds consisting of 1-decanoyl-*sn*-glycero-3-phosphorylcholine, 1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine, hexadecylphosphocholine, Lysophosphatidic acid, palmitate, N-(4-hydroxyphenyl)retinamide, phosphatidyl-3,4-dihydroxybutyl-1-phosphate, phosphatidylserine, sphingosine-1-phosphate, and sulfoquinovosyldiacylglycerol.

36. The composition of claim 34, wherein the inhibitor of cardiolipin synthesis is a polynucleotide having a sequence antisense to the coding sequence of an enzyme in the cardiolipin synthesis pathway.

37. The composition of claim 36, wherein the enzyme is selected from the group of enzymes consisting of phosphatidylglycerophosphate synthase, phosphatidylglycerophosphate phosphatase and cardiolipin synthase.

38. The composition of claim 34 also comprising an antineoplastic agent.

39. The composition of claim 38, wherein the antineoplastic agent is selected from the group of antineoplastic agents consisting of mitoxantrone, taxanes, paclitaxel, camptothecin, camptothecin derivatives, topotecan, gemcitabine, vinorelbine, vinblastine, anthracyclines, adriamycin, capecitabine, docetaxel, didanosine (ddl), stavudine (d4T), antisense oligonucleotides, antibodies, immunotoxins, hydroxyurea, melphalan, chlormethine, extramustinephosphate, uramustine, ifosfamide, mannomustine, trifosfamide, streptozotocin, mitobronitol, mitoxantrone, methotrexate, 5-fluorouracil, cytarabine, tegafur, idoxide, taxol, daunomycin, daunorubicin, bleomycin, amphotericin, carboplatin, cisplatin, BCNU, vincristine, mitomycin, doxorubicin, etoposide, histernine dihydrochloride, tamoxifen, cytoxan, leucovorin, oxaliplatin, irinotecan, raltitrexed, epirubicin, anastrozole, proleukin, sulindac, EKI-569, erthroxyllaceae, cerubidine, docetaxel, cytokines (e.g., interleukins), ribozymes, interferons, oligonucleotides, and functional derivatives and combinations thereof.



1/2

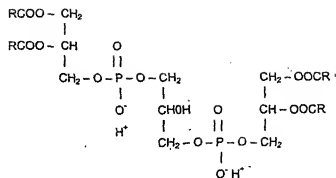


Figure 1: Cardiolipin Structure

2/2

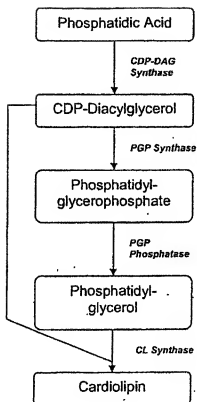


Figure 2: Cardiolipin Biosynthetic Pathway

229579.ST25  
SEQUENCE LISTING

<110> Neopharm, Inc.  
 Haris, Jamil  
 Ahmad, Moghis U.  
 Ahmad, Imran

<120> Methods of Inducing Apoptosis and Inhibiting Cardiolipin  
 Synthesis

<130> 229579

<150> US 60/480,669

<151> 2003-06-23

<160> 6

<170> PatentIn version 3.2

<210> 1

<211> 2130

<212> DNA

<213> Homo sapiens

<400> 1  
 gctccggcgg cttcgctgct agctcgcggc gacgtcgggc cgattttccc aggatgacag 60  
 agctgagga gaggggtggc catgagccgg ttgcgccacc cgaggacaag gagtcagagt 120  
 cagaagcaaa ggtagatgga gagactgcat cggacgggca gaatccgcac 180  
 cctcgccagt cttcgagat gataccggc aggtcctcaa tagggccctt tccaacttgt 240  
 cttcaagatg gaagaactgg tgggtgagag gcatcctgac ttggccatg attgcatttt 300  
 tcttcatcat catttacctg ggaccaatgg ttttgatgat aatcgtgat tgcgttcaga 360  
 ttaagtgttt ccatgagata atcactattg gctacaacgt ctaccactca tatgatctgc 420  
 cctggttcag gacgctcagc tggactttc tctgtgtgt aaactatttc ttcattggtg 480  
 agacagtgc ggattacttc ttcaccctgg tccagagaga agagcctttg cggattctca 540  
 gtaaatacca ccggttcatt tcctttactc tctatctaag aggattctgc atgtttgtac 600  
 tgagtctggt caagaagcat tatcgactgc agttctacat gtttggtcgg acccatgtga 660  
 cattgtgat tgttgtaaca cagtcacatc ttgttatcca caacctattt gaaggaatga 720  
 tctggttcatt tgtcccata tcttgtgtga tctgtaatga catcatggcc tatatgtttg 780  
 gctttttctt tggtcggacc cactcatca agctgtcccc gaagaagacc tgggaaggct 840  
 tcattggggg cttctttgct actgtggtgt ttggccttct gctgtcctat gtgatgtcgg 900  
 ggtacagatg ctttgctgc cctgtggagt acaacaatga caccaacagc ttcactgtgg 960  
 actgtgagcc ctcggaactg tttcgctgc aggaagtaca cattcctggg gtgatccagt 1020  
 cagtcattgg ctggaaaacg gtccggatgt accccttcca gattcacagc atcgctctct 1080  
 ccacccttgc ctgcctcatt ggccccttgg gaggattctt cgcaagtgga ttcaaacgag 1140  
 cctttaaata caaagacttt gccaatacca ttctggcca tggaggcatc atggatcgct 1200  
 ttgactgcca gtatctgatg gccacccttg tcaatgtata catgccagt tttatcagag 1260

229579.ST25

```

gccctaacc aagcaaaactg attcagcagt tcctgacttt acggccagat cagcagctcc 1320
acatcttcaa cagcgtgcgg tctcatctga tcgacaaagg gatgctgaca tccaccacag 1380
aggacgagta ggggccaccc agggccagga gaacaggaac agaactgagc aggggcaggt 1440
ctccaagaaa tcctgcttg gagctgcaga agggttgcct tctgtaggtc ggaggaaatgg 1500
aggcttacta accaggtaa cttctatgc atccacacca aaatcctgca gaatgtaagt 1560
aagctctgct ttataagatg ggttcacctt catcgagac tgaaagtttc agtttttatt 1620
tttttcagaa agcagaaaaa attatttata atagtctgga gaaaaaacac actgtaatat 1680
ttcaagtgta tgcagtagaa tgtactgtaa ctgagccctt tcccacatgt ctaggctcca 1740
atgtctctg taggtccacc taactgtgtg ttttcagggg caatgccatc catgtttgtg 1800
ctgtagactt gctgctgctg aatcctttct ggggactttc tcacgaggca gggagcagag 1860
ggctctctgt tcatgcaccc ttgtcctgaa caccatgta gctgctgtgt tgtgtatata 1920
ttactcttaa gaggagtgtg tgtgtctgtg tttgttttaa aagtcactta tttcttacag 1980
tgatttcaat tgcacatga ctcttctact aaaaccacaa agtcttgctt aaaactatgg 2040
aaaacctaac ctgattagag ctttgactat ttggaagatt aaatgcacac tttttatata 2100
aaaaaaaaa aaaaaaaaaa aaaaaaaaaa 2130

```

```

<210> 2
<211> 2177
<212> DNA
<213> Homo sapiens

```

```

<400> 2
ctgcggcggg acccgtgttc tggaggcgac tgctgggcct cctgcctggc cgccaggggc 60
tggccgcgct cctgggacgc ctgtccgacc gcctcggcag gaaccgggac cgccagcgca 120
ggaggtcacc atggctgtta ttggctccct tgctgtcccc agctgttccc caggtcacct 180
ccccaccttg ctgcctgtgt ccagaaggcg tgcaccggtt ccagtggatc agaaacctgg 240
ttccagaatt tggagtcttc agttctcacg ttaggggtgt ttcttccccc gcagagtttt 300
tcgagctcat gaaggggcag ataagagtag ccaagaggcg ggtcgtgatg gcatccctct 360
acctggggac aggtcctttg gaacaggagc tgggtggact cctgaaaagt actctagaaa 420
agtcactcca agcaaaagt ctttcaaata tcaaggctct cattctctta gacttcacgc 480
ggggctcacg aggtcggaaag aactcccgca caatgctgct cccactcctg cggagggttc 540
cagagcaggt ccgagtctcc ctctttcaca cgccgcacct ccgtgggctg cttcggctcc 600
tcattccctga gcgcttcaac gagaccatcg gcctccagca cattaaagtg tacctcttcg 660
acaacacgct catcttgagc ggtgcaaac tgagtgaact ctacttcacc aaccgccagg 720
accgctactg gttcttgacg gactgtgcgg agattgccga ctcttcacg gagctgggtg 780
acgcggtggg ggatgtgtcc ctgcagctgc agggggacga cacggtgcag gtggtggatg 840
ggatggtgca tccttcaaaa ggggaccggg ccgagtactg caaggcagcc aataagaggg 900

```

229579.ST25

```

tcatgtagt gatcaactca gccaggaccc gccagcagat gctgcatgcc cagaccttcc 960
acagcaactc tcttttgacc caggaagatg cagcagctgc tggggatcgc agaccagccc 1020
ctgacacctg gatttatccg ctgattcaga tgaagccctt cgagaattcaa atcgatgaga 1080
ttgtcactga gacctgtgtg actgagggcg agcgcggggc aaaggcttac ctaccacctg 1140
gctatttcaa cctgaccag gccatcatgg acctggtctt gggcactcgg gctgagtacc 1200
agatctctgt ggcctcacca gagggaatg gcttctttgg ggccaagggg gtggccggcg 1260
ccatccacgc ggcctatgtg cacatcgagc gacagtctct cagtgggtg tgcagcctgg 1320
gacagcagga gcgggtccag ctccaggagt actggcggag gggctggagc ttccacgcca 1380
aaggcctctg gctgtacctg gcaggagca gctgcacctg tctcacgctg attggtcttc 1440
ctaattttgg gtacaggta gttcaccggg acctggaggc ccagattgcg atcgtagcgg 1500
agaaccaggc cctgcagcag cagcttcacc aggaagcaaga gcagctctac ctgaggtcag 1560
gtgtggtgtc ctctgccacc ttcgagcagc cgagtcgcca ggtgaagctg tgggtgaaga 1620
tggtgacttc actgatcaag aacttcttct gaggacagac aggaatggcc ttgatgaaga 1680
tgacaggcat gggcggggtc agctctttca gccgcgcttc agcgatgact ccagctctggg 1740
tgtcccagcg agcccttgca gggacagtat ggctgagggt caggtgtgct gccagtaagt 1800
gagggagggg ctggcaggaa ggggtgggtc ctcacactcc ccgccctctg cagagctggg 1860
ctctacccca aaaggcttca ggccagctgc cacagctgga agcagaggcc ttctgaggtg 1920
atggcctgca tgttgaact acccgtccc gctgggctca aggaacagct cagctaaagc 1980
cctcggttcc catccgttta aatctgtggc attttcagag cctcatctgt cagccttaat 2040
gtcagtgcca ggaagtcata actccagcta aaaattacag agtaaaagttc cctgattctt 2100
aatgtgtaat gtctgcaccta tgtgtacata cacaataaa ttatacatct gtgcatataa 2160
aaaaaaaaa aaaaaaa 2177

```

```

<210> 3
<211> 791
<212> DNA
<213> Mus musculus

```

```

<400> 3
agctacacag cttgtgcat ggactctgag gtcagcaacg gttccggcct gggggccaag 60
cacatcacag acctgtctgt gttcggcttt ctccaaagct ctggctgtac tcgccaagag 120
ctggagggtc tgggtcggga actgcctgtg caagcttact gggaggcaga cctcgaagac 180
gagctgcaga cagacggcag ccaggccagc cgcctcttca accaaggaag aatagagcca 240
gattctgaaa gtcaggaaga aatcatccac aacattgcca gacatctcgc ccaaataggc 300
gatgagatgg accacaacat ccagcccaca ctggtgagac agctagccgc acagttcatg 360
aatggcagcc tgtcggagga agacaaaagg aactgcctgg ccaagccctt tgatgaggtg 420
aagacagcct tccccagaga catggagaac gacaaggcca tgctgataat gacaatgctg 480

```

229579.ST25

ttggccaaaa aagtggccag tcacgcacca tctttgctcc gtgatgtctt ccacacgact	540
gtcaacttta ttaaccagaa cctattctcc tatgtgagga acttggttag aaacgagatg	600
gactgaggag cccgcacaag cccgatggtg aactgctcc cagaggaaac gcgaccatgg	660
aaagaccttg gcttgaagac aggtccca gaacagctgt ctccctattt ccaggtggtg	720
ggaaccccaa gctggtgatt cactggacat ctctgcgttc agcttgagtg tatctgaaga	780
gtttacgcg g	791

<210> 4  
 <211> 2575  
 <212> DNA  
 <213> Homo sapiens

<400> 4

ggaggaggaa gcaagcgagg gggctggttc ctgagcttgc caattcctgt gtcgcttctt	60
gggctccag cctgccggt cgcatgatcc ctccggcgg agctggttt ttgccaagcc	120
accgcgaggg cggtgagtt accggcatcc ccgcagccac ctctctctcc gacctgtgat	180
acaaaagatc ttccgggggc tgcacctgcc tgctttgccc taaggcggat ttgaatctct	240
ttctctcctc tcagaatctt atcttggtt tgatcttag aagagaatca ctaaccagag	300
acgagactca gtgagtgagc aggtgtttt gacaatggac tggttgagcc catccctatt	360
ataaaaaagt ccagagcaa ccgggagctg gtggttgact ttctctcta caagctttcc	420
cagaaaaggt acagctggag tcagttagt gatgtggaag agaacaggac tgaggcccca	480
gaaggggactg aatcgagat ggagacccc agtgccatca atggcaacc atcctggcac	540
ctggcgacac gccccgcggt gaatggagcc actggccaca gcagcagttt ggatgcccg	600
gaggtgatcc ccatggcagc agtaaaacaa gcgctgaggg aggcaggcga cgagtttgaa	660
ctgcggtacc ggcgggcatt cagtgcctg acatccagc tccacatcac ccagggaca	720
gcataatcaga gctttgaaca gtagtgaat gaactcttcc gggatggggt aaactggggt	780
cgcatgttg cttttttctc ctctggcggg gcactgtgcg tggaaagcgt agacaaggag	840
atgcaggatg tggtagctg gatcgagct tggatggcca cttaacctga tgaccaccta	900
gagccttgga tccaggagaa cggcggtctg gatactttg tggaaactta tgggaacaat	960
gcagcagccg agagccgaaa gggccaggaa cgcttcaacc gctggtctc gacgggcatg	1020
actgtggccg gcgtggttct gctgggtcct ctcttcagtc ggaatgacc agacactgac	1080
catccactct accctccac cccctctct gctccaccac atcctcgtc cagcgcctat	1140
tgccaccagg agaaccacta catgcagccc atgccacact gccatcaca gggttgggcc	1200
cagatctggt cccttcgagc tagttttcta gaatttatca cacttctgtg agacccccac	1260
acctcagttc ccttgccctc agaattcaca aaatttcac aaaatctgtc caaaggaggc	1320
tggcaggatg ggaagggtt gtggctgggg gcaggagggc cctacctgat tggtgcaacc	1380
cttaccctct agcctccctg aaaaatgttt tctgccagg agcttgaag ttttcagaac	1440

229579.ST25

ctcttcccca gaaaggagac tagattgcct ttgttttgat gtttgtggcc tcagaattga	1500
tcattttccc cccactctcc ccacactaac ctgggttccc ttcccttcca tccctacccc	1560
ctaagagcca tttaggggcc acttttgact agggattcag gctgcttggg ataaagatgc	1620
aaggaccagg actccctcct caccctctga ctggctagag tcctcactcc cagtcctaat	1680
gtcctccaga agcctctggc tagaggccag cccaccacag gagggagggg gctatagcta	1740
caggaagcac cccatgcca agctagggtg gcccttgca ttcagcacca cctagtcccc	1800
ttccctcccc tggctcccat gaccatactg agggaccaac tgggcccaag acagatgccc	1860
cagagctgtt tatggctca gctgcctcac ttctacaag agcagcctgt ggcattctttg	1920
ccttgggctg ctctctatgg tgggttcagg ggactcagcc ctgaggtgaa agggagctat	1980
caggaacagc tatgggagcc ccagggtctt ccctacctca ggcaggaaag gcaggaaaga	2040
gagcctgtct catggggctg ggtagggtg actagaagg ccagtcctgc ctggccaggc	2100
agatctgtgc cccatgcctg tcagcctgg gcagccagc tgccaaggcc agagtggcct	2160
ggccaggagc tcttcaggcc tccctctctc ttctgtcca ccttggcct gtctcatccc	2220
caggggtccc agccaccccg ggctctctgc tgtacatatt tgagactagt ttatttccct	2280
tgtgaagatg atatactatt ttgttaagc gtgtctgtat ttatgttga ggagctgtctg	2340
gcttgcaagt cgcgtgcacg tggagagctg gtgcccgag attggacggc ctgagtctcc	2400
ctccctgccc ctggtccagg gaagctggcc gagggctcct gctcctgagg ggcattctgc	2460
cctcccccac ccccccacc acacttggtc cagctctttg aaatagtctg tgtgaaggtg	2520
aaagtgcagt tcagtaataa actgtgttta ctcagtgaaa aaaaaaaaaa aaaa	2575

<210> 5  
 <211> 765  
 <212> DNA  
 <213> Shigella flexneri serotype 2a str. 2457T

<400> 5 atgctgtcga ttgccgcagc taccgcagtg ggagctgcac tattgcttgt catgccagta	60
gccgtatgga tttctggctg gcgttggcaa cctggagaac aaagtggctg actaaaagcg	120
gctttttggg ttactgaaac tgcaccacag ccctggggcg tcattacaca ttgatttttg	180
ttcggctggg ttctctgggt tctgcgtttt cgcattaagg ctgcctttgt attatttgcc	240
attctggcgg cggcaatcct tgttggaaca ggcgttaaat cctggatcaa agacaaagta	300
caggaaccac gaccttttgt tatctgggtg gaaaaaacac atcatattcc ggttgatgag	360
ttctacactt taaagcgagc agaacgcgga aatctagtaa aagaacagtt ggcgtgaagag	420
aaaaatatcc cacaatattt gcgttcacac tggcagaaag agacgggggt tgcctttcct	480
tccggtcaca cgatgtttgc tggcagttgg gcactgctgg ccgttgggtt gctgtggccg	540
cgctcggcaa cgtaaacatt tgctatcttg ttggtctggg caacgggagt catgggaagc	600
cgctctctgc tcgggatgca ttggccacgc gatctgtggg tagctacgtt gatttcgttg	660

229579.ST25  
 gcgcctggtg cggtggcaac gtggcttgcg caacgaattt gtggccatt aacaccact 720  
 gcggaagaaa atcgcgaaat tgcacaacga gaacaagaaa gttaa 765

<210> 6  
 <211> 1440  
 <212> DNA  
 <213> Oceanobacillus iheyensis HTE831

<400> 6  
 atgggaataa cctctttgtt attaggacta acatttgttt taaatattgc tttagctatt 60  
 tcaattattt ttcttgaacg taaagatcca acgtcctctt gggcatgggt aatggctcct 120  
 ttatttatac ccatacttgg attctttctt tatttaatat ttggcaaac aatcagtaat 180  
 agaagattt ttcttggga taagaagagc cgtttgggag taaagacaac agtcaatct 240  
 caattgagac tcttagaaga aaatcaattt gaatttaatc aaccagacct tatcgagcat 300  
 aaggatcttg tatatttaca ttgaaaaat gatgaagcaa ttatatacaca gaataatggt 360  
 gtgatattt ttacagatgg tcaaacgaaa ttgatgctt tgctagaaga tatagaaaaa 420  
 gcgaaaaaac atatacatat tcagtactat attatgcgca gtgatggctt aggaaatagg 480  
 cttgcagaca tgctaataaa aaaagtaaat gaagggtgag aagttagagt tttatatgat 540  
 gatatgggat caagatcggt aaaaaacagt tatataaaac gtttaaaaag agcaggagtg 600  
 atggtcgagg cattcttccc atctcgattc atagtcattt tcaagattaa ttatcgaaat 660  
 caccgtaagc tagcgattat tgatggatat attgatatt taggtggttt taatgtaggc 720  
 gatgaatatt tagggattaa taaaaagttt ggatattgga gagacaccca tcttcgtgtg 780  
 attggagatg cggtaacaaag tatgcaaaaca cgttttatcc tagattggaa ccaagcatcg 840  
 agggatacta ttttatataa tgaagattat tatcaaacag tatctgctgg aaatgtcgg 900  
 atgcaaatg ttactagtgg tcctgattca gaatatgaac aaatcaagaa tggttatata 960  
 aagatgatca tggaggcaaa cgatttatatt tgtatccaaa ctcttattt tatccggat 1020  
 gaaagtttaa gggatgcatt aaaaattgca gtattatctg gggatcatgt taaattatg 1080  
 ataccgaaca aacctgatca tccatttgta tattgggcaa cattatcgta ttgtggcgat 1140  
 ttaattcaag ctgtgctga aatatttatt tatcagaatg gatttttaca tgcataaaca 1200  
 atcattgtgg atggtaggat agcttcagtt ggaacagcta atattgatgt acgtagtctc 1260  
 cgacttaatt tcgaagtaaa tggcttcttg tatgattctg aagtagtgaa tcggcttcaa 1320  
 aatgagttcg acgcagattt ggagaaatct acgcaaatga caaggaaact ttatgatcaa 1380  
 agatcgatag gaatccggtt taaagaatct atatccaggt tgatttcacc tgttttataa 1440